

## 1. Introduction

Peptides are widely used in the fields of clinical diagnosis, disease treatment, nutrition, etc. In order to achieve the accuracy, consistency, and equivalence of peptide measurement results, CCQM PAWG has already organised a series of international comparisons (e.g. CCQM-K115) on purity assessment for peptide. All the peptides studied by PAWG have a linear or low cross-linked structure [1]. Notably, high cross-linked peptides containing multi- intra and/or inter-molecular disulfide bonds have a certain resistance to oxidants and proteases *in vivo*, thus they show superior metabolic stability and biological activity properties compared to a linear analogue peptide. Given these abovementioned advantages, this kind of substances are increasingly used as a source for peptide drug research and development. Currently, some disulfide-rich peptides have been officially approved as therapeutic medicines, such as the  $\omega$ -conotoxin ziconotide (Prialt). Given the medicinal peptide with unexpected disulfide connectivity might not have efficacy, even induce immunogenicity, it is required to develop a reliable method assigning disulfide connectivity. Meanwhile, structurally related impurities generated during peptide synthesis, storage, and transportation processes are highly required to be accurately determined. In this study, novel methods have been developed for determination of structurally related impurities, and assignment of disulfide connectivity for ziconotide, a chosen mode for disulfide-rich peptide. Its amino acid sequence is CKGKGAKCSRLMYDCTGSCRSKGK-NH<sub>2</sub>, in which three disulfide bonds are formed with connectivity of C1-C16, C8-C20, C15-C25.

## 2. Results

### 2.1 Assignment for Disulfide Connectivity in Ziconotide

6 cysteine moieties presenting in ziconotide molecule forms 3 disulfide bonds, which could produce 15 possible disulfide isoforms with different disulfide connectivities. A relatively simple method based on partial reduction without alkylation has been developed to assign disulfide connectivity for ziconotide. After partial reduction reaction, the partially reduced ziconotide products containing one disulfide bond were analyzed by liquid chromatography tandem mass spectrometry [2]. The information about the sequence uninvolved in the cyclic structure was clearly obtained by tandem mass spectrometry, which reveals the connectivity of the disulfide bridges forming the cyclic structure. Based on the results of only two partially reduced products, accurate connectivity of all disulfide bridges in ziconotide was obtained.

a) After 4 h reduction reaction with TCEP, the reduced products were analyzed using LC-MS. Reduction products were briefly interpreted by using their exact masses and isotope distributions. Three partially reduced products containing one disulfide bond ( $m/z = 882.05$ ,  $z = 3$ ) were observed with retention times of 27.4 min, 30.5 min, and 34.5 min.

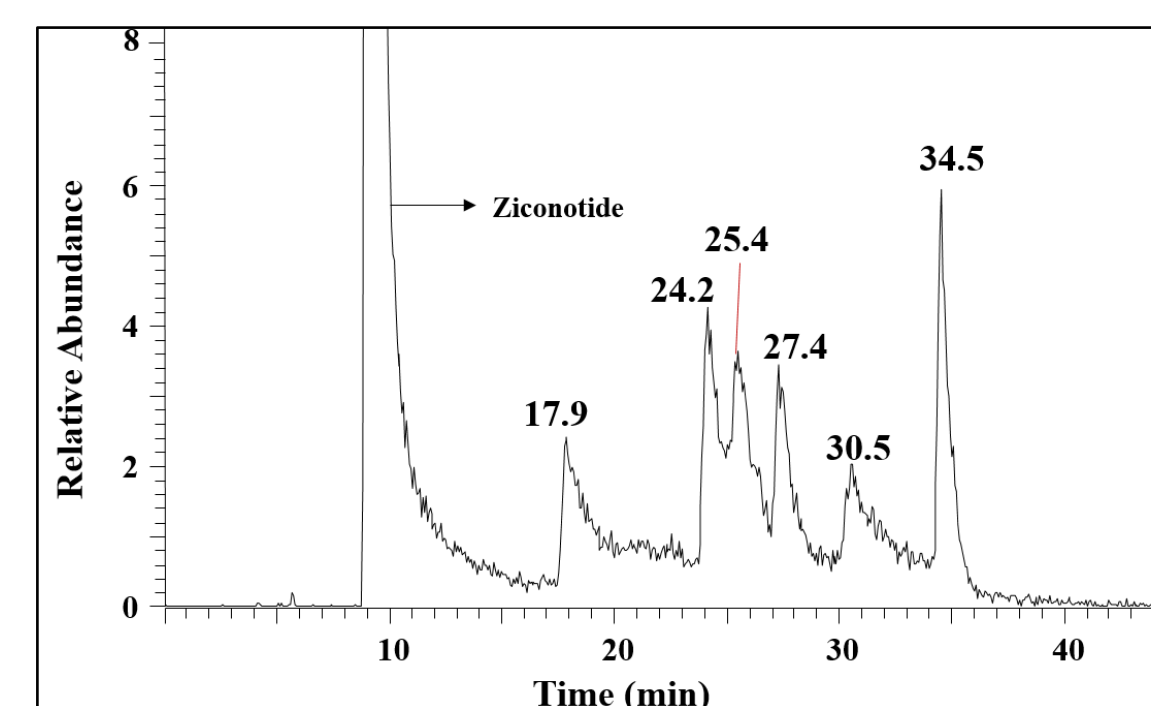


Figure 1a Total ion chromatograms of partially reduced ziconotide after ziconotide reacting with TCEP

b) b4 and y21 were selected as characteristic ion pairs between Cys1 and Cys8. b12 and y13 were selected as characteristic ion pairs between Cys8 and Cys15. b15 and y10 were selected as characteristic ion pairs between Cys15 and Cys16. b182+ and y7 were selected as characteristic ion pairs between Cys16 and Cys20. b24 was selected as characteristic ion between Cys20 and Cys25.

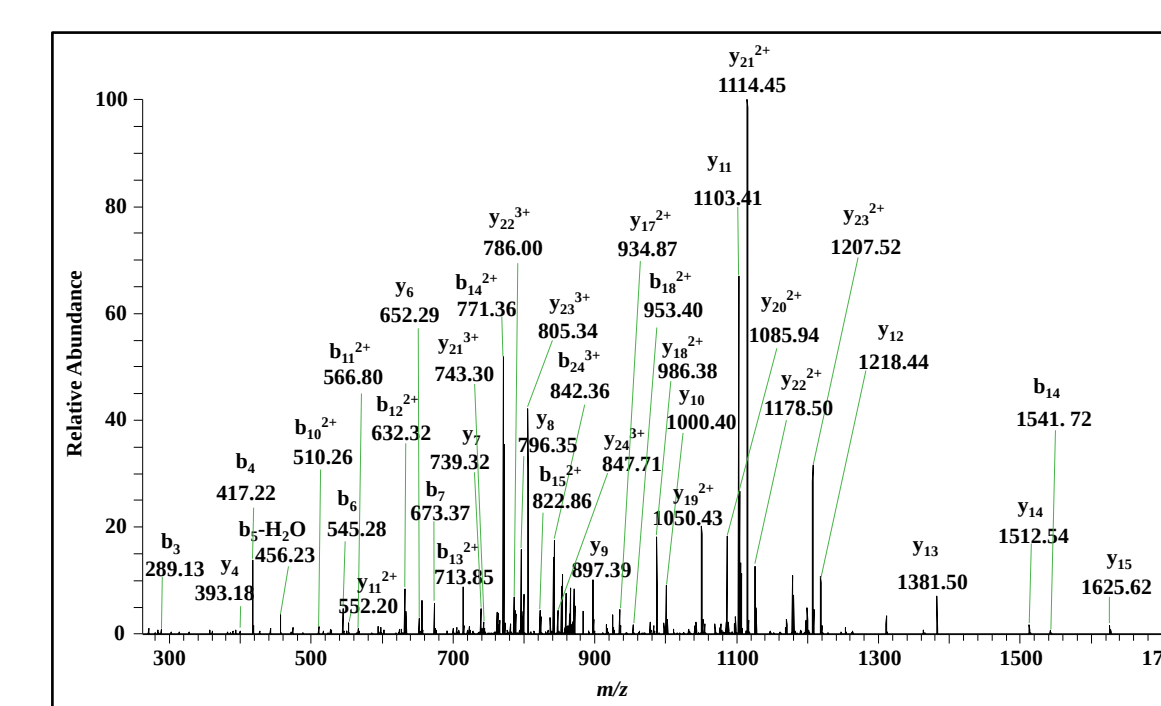


Figure 1b MS<sup>2</sup> spectra of complete reduced ziconotide upon collision-induced dissociation

c) The presence or absence of the characteristic ions in the MS<sup>2</sup> spectra of partially reduced products could be easily observed. For example, b<sub>4</sub> ions generated from which have same mass as b<sub>4</sub> ions generated from complete reduced ziconotide, indicating Cys1 is not involved in the cyclic structure. Finally, the S-S connectivity in reduction product 1 is Cys8-Cys20.

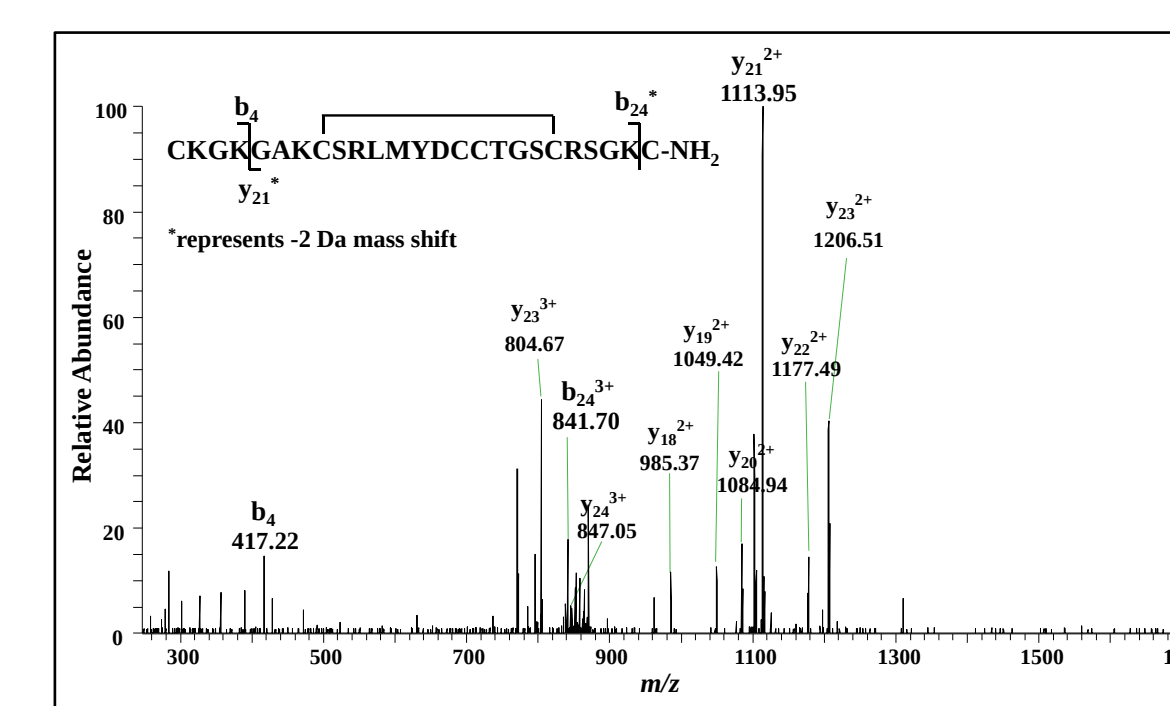


Figure 1c MS<sup>2</sup> spectra of partially reduced ziconotide containing one disulfide bond at retention times of 27.4 min

d) Cys1 and Cys8 are not involved in a cyclic structure. y<sub>13</sub> and y<sub>21</sub> ions have +2 Da mass shift, indicating presence of one disulfide bond. Absence of characteristic ions (e.g. b<sub>15</sub>, y<sub>10</sub>, b<sub>18</sub>, y<sub>7</sub>, and b<sub>24</sub>) proves Cys15, Cys16, Cys20, and Cys25 are involved in a cyclic structure, determining disulfide bond connectivity as Cys15-Cys25.

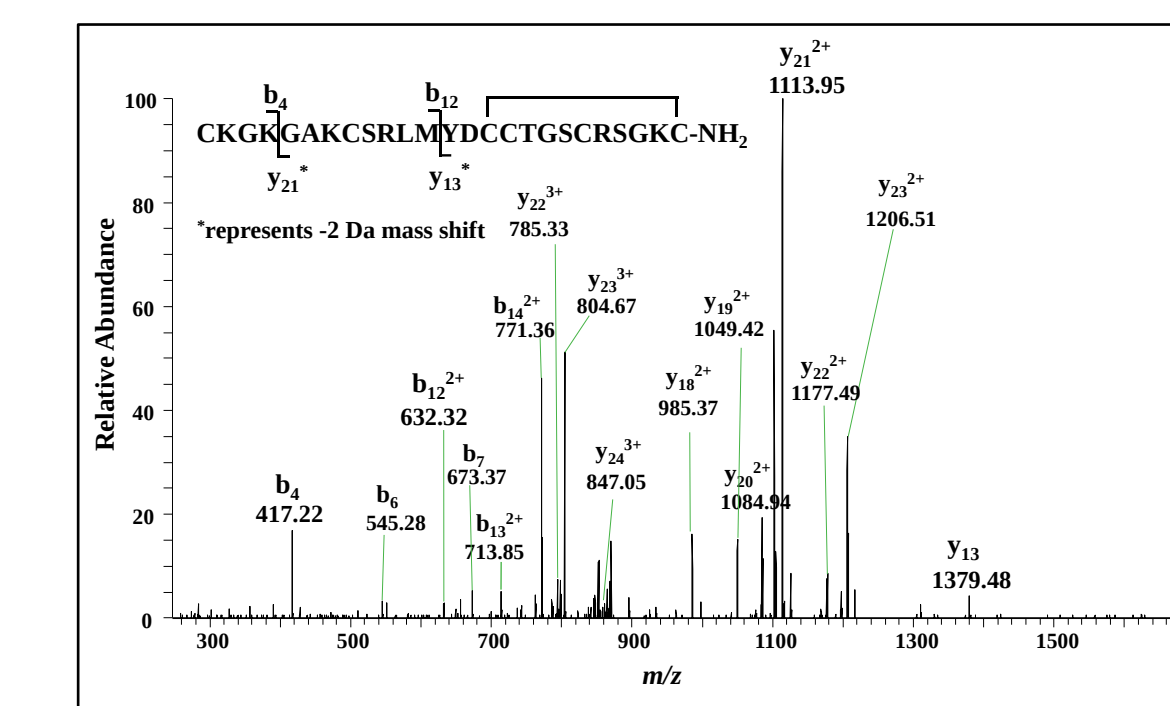


Figure 1d MS<sup>2</sup> spectra of partially reduced ziconotide containing one disulfide bond at retention times of 34.5 min

e) Based on two identified disulfide bonds as Cys15-Cys25, Cys8-Cys20, the remaining two cysteine moieties (e.g. Cys1, Cys16) form third disulfide bond as Cys1-Cys16. The partially reduced product containing one disulfide bond of Cys1-Cys16 was not clearly determined in this experiment, probably because Cys1-Cys16 is located on the ziconotide molecule surface.

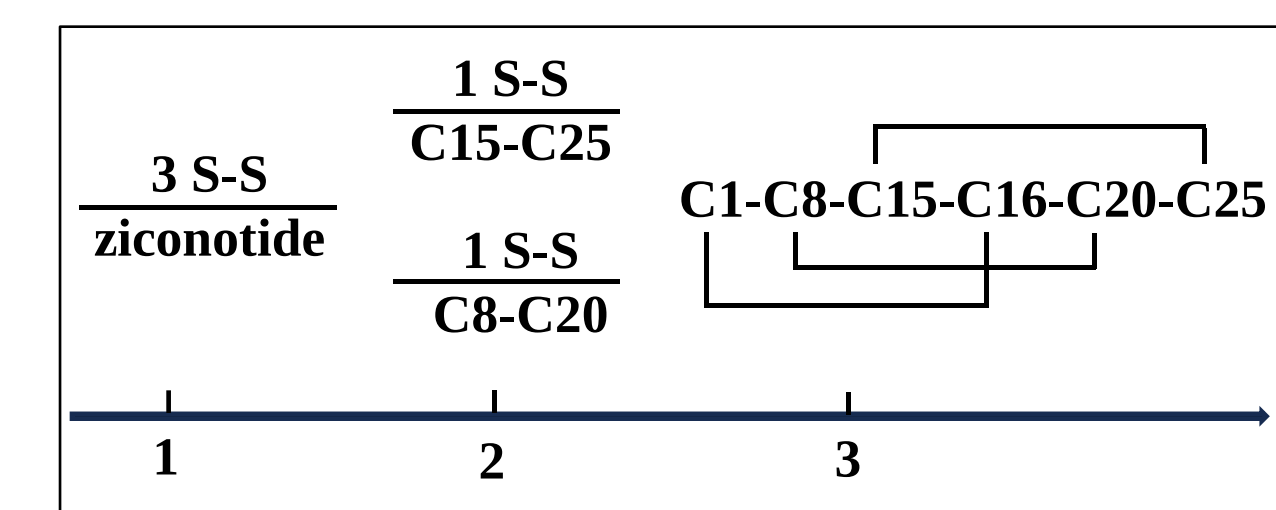


Figure 1e Assignment for the third disulfide bond

## 2. Results

### 2.2 Determination of structurally related impurity in ziconotide

Novel methods based on tandem mass spectrometry have been established characterizing structurally related impurities in a ziconotide. By the LC-hrMS<sup>2</sup> method developed, seven ziconotide impurities (e.g. [Mox<sup>12</sup>]ziconotide and its isomer, [dehydro-A<sup>16</sup>] ziconotide, ziconotide-9S, ziconotide +  $\beta$ A10, two ziconotide isomers) were separated and characterized [3]. A total mass fraction of related substances in the ziconotide study material was determined to be 206 mg/g  $\pm$  28 mg/g ( $k = 2$ ). Among these impurities, ziconotide isomer 2 stands out as a critical component (>100 mg/g), which significantly impacts the peptide purity and quality of the ziconotide. An MS<sup>2</sup>-IMS method was developed to locate the isomerization site for a disulfide-rich peptide isomer without requiring synthetic standards. Finally, ziconotide isomer 2 was identified as [iso-Asp<sup>14</sup>]ziconotide. Despite [iso-Asp<sup>14</sup>]ziconotide has not been reported possessing toxicity or activity, the presence of such high level impurity still leads to concerns about the efficacy of ziconotide due to low purity. The methods established here could also benefit for in-depth research on related substances for other disulfide-rich peptides and peptide purity assignment using mass balance approach (e.g., summation of impurities), amino acid analysis, etc.

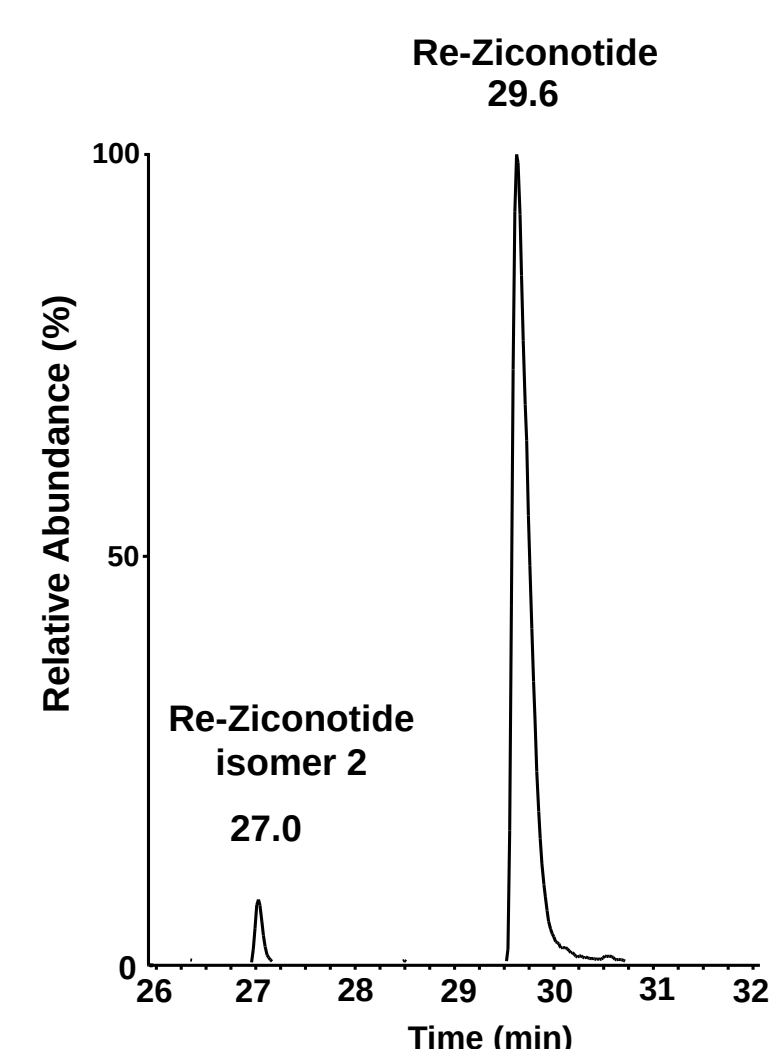


Figure 2a Extracted ion chromatogram of reduced ziconotide

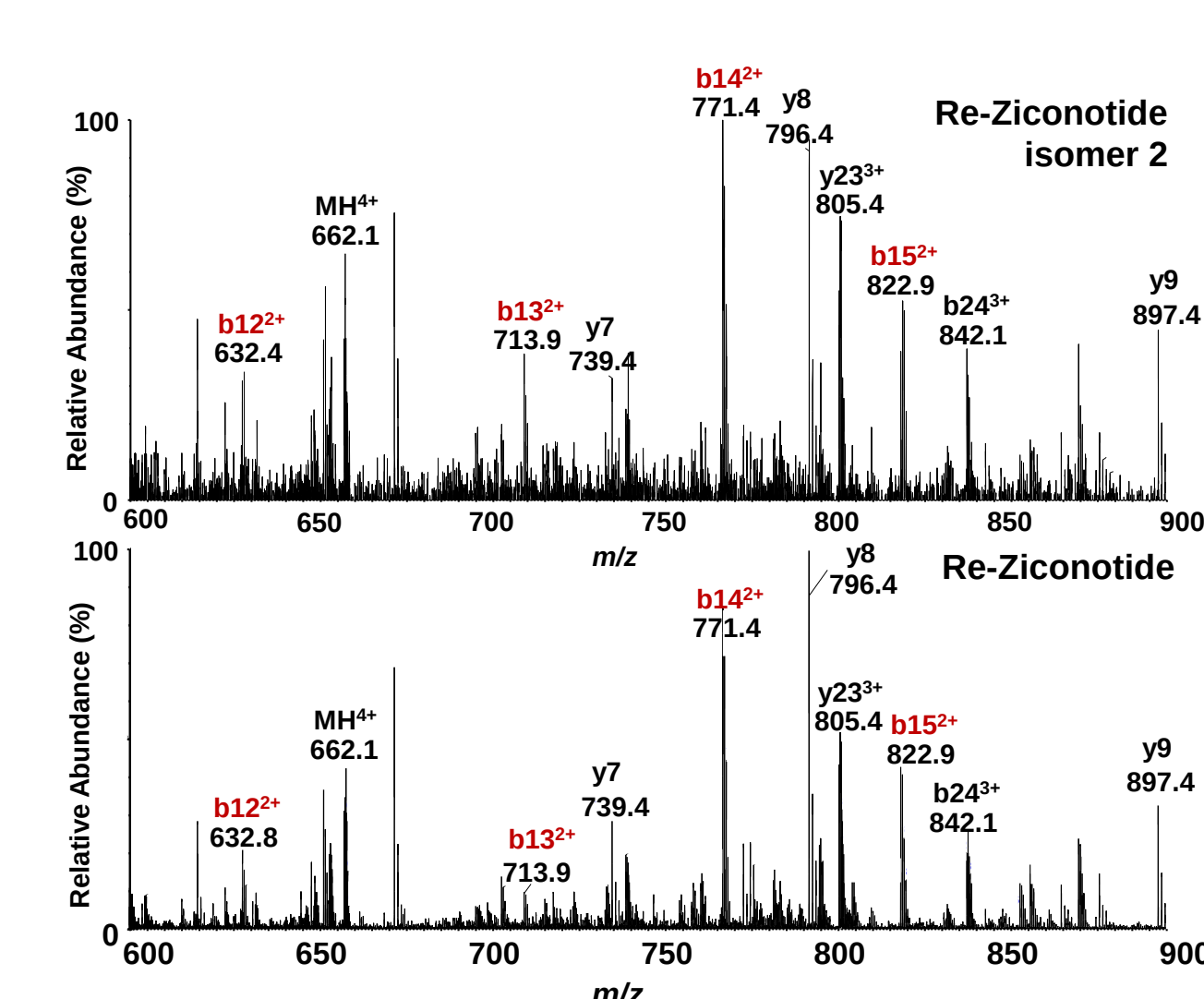


Figure 2b Tandem mass spectra of re-ziconotide isomer 2 and re-ziconotide

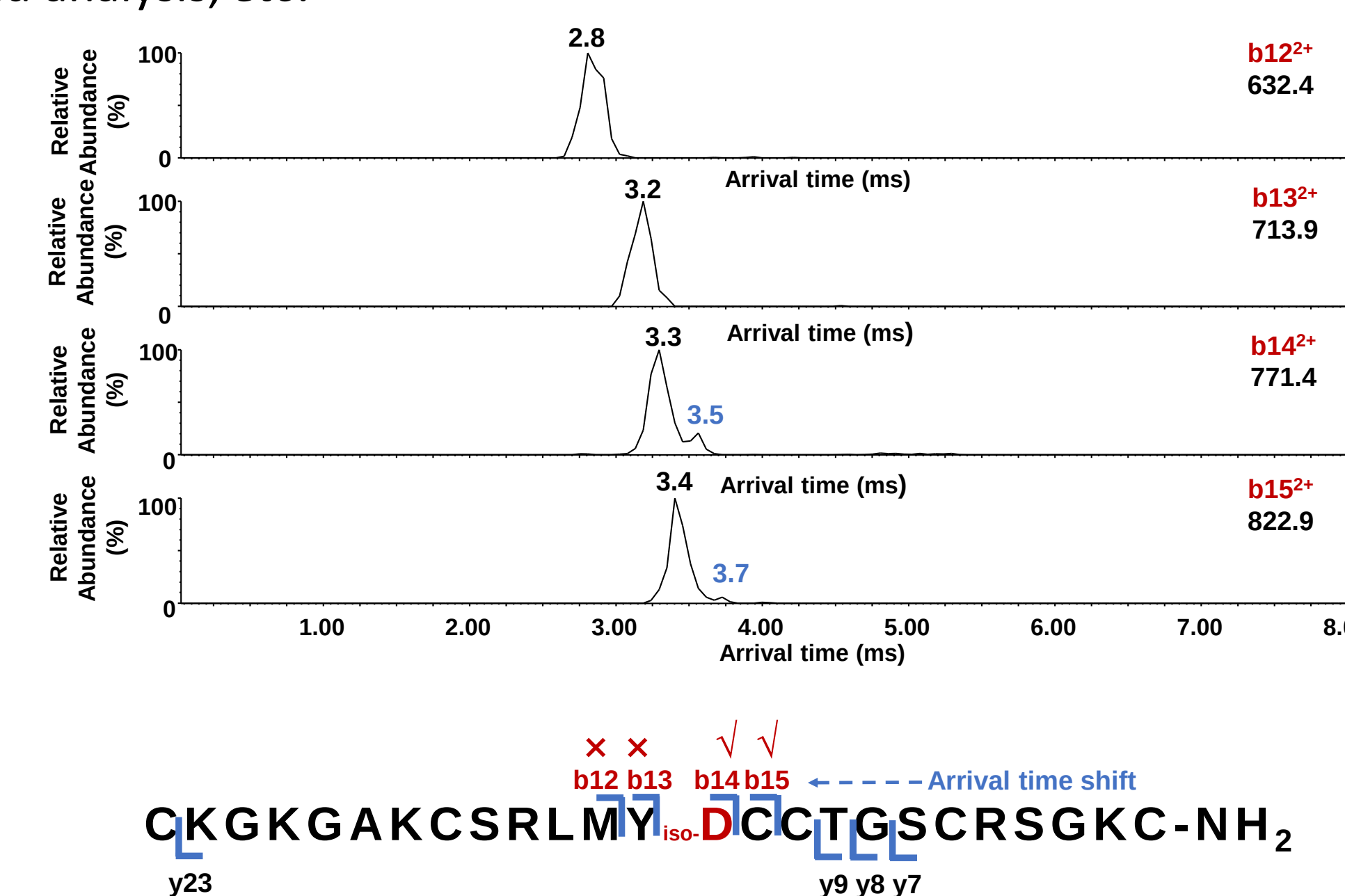


Figure 2c Isomerization site located by arrival time shift (✓: shift; ×: without shift)

## 3. Conclusion

Disulfide bond, formed by covalently crosslinking two thiol groups (-SH) in the side chains of two cysteine (Cys) moieties within or between peptides or proteins, is a very important post-translational modification. Compared with a linear peptide, a peptide containing disulfide bonds have certain resistance to oxidants and proteases from the environment, so they have better metabolic stability and biological activity. Therefore, this kind of peptides are increasingly used as a medicine. Ziconotide is the first member receiving approval for intrathecal therapy of severe chronic pain in the disulfide-rich neuroactive conopeptide (conotoxins) family. Given the bond energy of disulfide bond is larger than that of amide bonds in the backbone, conventional tandem mass spectrometry (e.g., collisional induced dissociation, higher-energy collisional dissociation) cannot directly break disulfide bonds. Ziconotide molecule has three intramolecular disulfide bonds, rendering it difficult to be carefully characterized for disulfide connectivity assignment and structurally related impurity determination. In our study, novel methods based on mass spectrometry have been developed for intensive characterization of disulfide-rich ziconotide. This work will contribute to the peptide metrology, and thus provide valuable information for quality control of disulfide-rich peptide medicine.

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## Reference

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